ANALYSIS OF NOVEL BIOMARKERS FOR UNFAVORABLE BREAST CANCER PROGNOSIS

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– Till min kära familj

"If we knew what it was we were doing, it would not be called research, would it?"
Albert Einstein

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ABSTRACT

Breast cancer is the most common malignancy in women, and a major cause of mortality and morbidity despite the advances in diagnosis and treatment. The main challenge remains to identify novel biomarkers in order to improve existing treatment modalities. Ductal carcinoma in situ (DCIS) is considered as a direct precursor of invasive breast cancer. Therefore, it would be valuable to be familiar with the natural history of DCIS, including how it develops, and if it will progress to invasive breast carcinoma. Hence, the identification of biomarkers associated with DCIS progression may prevent the development of some invasive breast cancer tumors. The expression of S100A7 (psoriasin) has previously been identified in association with the transition from DCIS to invasive breast cancer. It has also been associated with unfavorable clinical outcomes, suggesting that psoriasin may play a role as a biomarker of aggressive malignant behavior. The first part of the thesis was conducted to investigate a potential role of psoriasin in breast cancer. We demonstrated that the reduction of intercellular adhesion molecule 1 (ICAM-1) by short hairpin RNA in mammary epithelial cells induced the expression levels of psoriasin, via the phospholipase C (PLC)-IP3 pathway, along with the oncogenic protein mucin1 (MUC1) **(Paper I).** We have shown that psoriasin contributes to the expression of vascular endothelial growth factor (VEGF) and elevated expression levels of psoriasin in mammary epithelial cells leads to increased endothelial cell proliferation in a paracrine manner through receptor for advanced glycation endproducts (RAGE) by promoting oxidative stress response **(Paper II).** In the second part of the thesis, we evaluated the expression levels of several candidate biomarkers in order to allow stratification of breast cancer tumors according to their aggressiveness. Previously, we performed analysis of gene expression in 97 primary invasive

diploid breast tumors and identified molecular gene signatures associated with poor clinical outcome. In **Paper III**, *CCNB2, CDCA7, ASPM, KIAA0101*, and *SLC27A2* were selected from these gene signatures. We studied their protein levels in association to patient clinical outcome in an independent cohort of 80 primary invasive breast tumors. Our data indicated that cytoplasmic CCNB2 may serve as a novel biomarker of unfavorable clinical outcomes over short-term follow-up in breast cancer**.** In addition, in a previous study, we performed gene expression analysis in 43 axillary lymph node negative tumors and identified 51 genes whose deregulated mRNA levels were significantly associated with unfavorable clinical outcome. Four candidate biomarkers; *GGH, FAAH, PIR* and *TAF5L* were selected among the identified 51-gene signature **(Paper IV).** We investigated their clinical impact in predicting breast cancer progression in an independent cohort of 80 primary invasive breast tumors. Our data suggest that elevated protein levels of GGH were associated with unfavorable prognosis and poor outcomes in breast cancer patients.

Our findings suggest that psoriasin, CCNB2 and GGH may be attractive targets for cancer therapy.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Petersson S, **Shubbar E**, Yhr M, Kovács A and Enerbäck C. Loss of ICAM-1 signaling induces psoriasin (S100A7) and MUC1 in mammary epithelial cells.
    Breast Cancer Res Treat. 2010; 125(1):13-25

II. **Emman Shubbar**, Jenny Vegfors, Maria Carlström, Stina Petersson and Charlotta Enerbäck, Psoriasin (S100A7) increases the expression of ROS and VEGF and acts through RAGE to promote endothelial cell proliferation
    Breast Cancer Res Treat. 2011; 134(1):71-80

III. **Emman Shubbar**, Anikó Kovács, Shahin Hajizadeh, Toshima Parris, Szilárd Nemes, Katrin Gunnarsdottir, Zakaria Einbeigi, Per Karlsson, and Khalil Helou, Elevated cyclin B2 expression in invasive breast carcinoma is associated with unfavorable clinical outcome
    Submitted, 2012

IV. **Emman Shubbar**, Khalil Helou, Anikó Kovács, Shahin Hajizadeh, Szilárd Nemes, Charlotta Enerbäck, and Zakaria Einbeigi, High levels of γ-glutamyl hydrolase (GGH) are associated with poor prognosis and unfavorable clinical outcomes in invasive breast cancer.
    Submitted, 2012

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CONTENT

ABBREVIATIONS

AH   Atypical hyperplasia

CCNB2   Cyclin B2

CGAP   Cancer Gene Anatomy Project

CI   Confidence interval

C-index   Concordance-index

DAB   Diaminobenzidine

DCIS   Ductal Carcinoma *in situ*

DSS   Disease Specific Survival

ECM   Extra Cellular Matrix

EGFR   Epidermal Growth Factor Receptor

ER   Estrogen Receptor

FFPE   Formalin-fixed, paraffin-embedded

FAAH   Fatty Acid Amide Hydrolase

GGH   γ-glutamyl hydrolase

GFP   Green Fluorescent Protein

HR   Hazard Ratio

ICAM-1   Intracellular adhesion molecule 1

IFNγ   Interferon-gamma

IHC   Immunohistochemistry

MMP13   Matrix metalloproteinase 13

MUC1	Mucin 1
NAC	N-acetylcysteine
NF-κB	Nuclear factor- kappaB
RB	Retinoblastoma
PIR	Pirin
PLC	Phospholipase C
RAGE	Receptor for Advanced Glycation Endproducts
PR	Progesterone Receptor
RFS	Recurrence Free Survival
ROS	Reactive oxidant species
SAGE	Serial Analysis of Gene Expression
shRNA	short hairpin RNA
TAF5L	TAF5-like RNA polymerase II, p300/CBP associated factor (PCAF)-associated factor, 65 kDa
QRT-PCR	Quantitative Real-Time PCR
VEGF	Vascular Endothelial Growth Factor

1 INTRODUCTION

Breast cancer tumors are highly heterogeneous in their morphology, biology, response to therapy and clinical course. It is the most common cancer among women in Sweden. In 2011, approximately 7000 women were diagnosed with the disease, which equates to around 18 women every day. It is estimated that one out of ten women in Sweden will develop breast cancer before the age of 75 years and approximately 1500 women will die from it annually [1]. However, despite the rising incidence of breast cancer, more women are surviving the disease than ever before. The relative 5- and 10-year survivals have improved in the present day to 89% and 79% respectively compared to 72% and 58% during the 1970's [2]. The improvements seen in survival rates are due mainly to advances in healthcare, including earlier and more accurate detection of breast cancer and more effective treatment [3]. In general, the regular treatment for breast cancer patients includes surgery in combination with radiotherapy and targeted therapy, endocrine, or chemotherapy. However, one in four of breast cancer patients will decease despite efforts for early detection and wide use of adjuvant systemic therapy. Therefore, identification of novel prognostic and predictive biomarkers for breast tumors is needed and remains a long awaited priority to enhance treatment.

Thus far, a complex interplay between several risk factors for the development of breast cancer have been proposed, including increasing age, gender, genetic risk factors (e.g.,*BRACA1* and *BRACA2* mutations*)*, life-style (e.g.*,* contraceptive pill and smoking), as well as reproductive factors including early age at menarche, late age at first birth and late menopause.

1.1 Hallmarks of cancer

In 2000, Hanahan and Weinberg published an article in which they described the "hallmarks of cancer". The changes that normal cells must obtain to avoid elimination by the body's defense mechanisms and to acquire a selective advantage over neighboring cells, formed a fundamental understanding of the biology and remarkable diversity of cancer [4]. In a follow-up article last year, the authors reviewed recent research on each of the cancer hallmarks noted in the original article and added two additional new hallmarks to bring the total number of hallmarks to eight [5]. The authors also described two hallmarks that enable tumorigenesis. These characteristics are common to all types of cancer despite if their genotypes differ and the order in which these hallmarks are required. Further down is a short description of the hallmarks of cancer.

Maintaining Proliferative Signals and Avoiding Growth Suppressors

When replacement cells in tissues are needed, essential growth signals from the surrounding microenvironment are released to activate trans-membrane receptors that trigger these cells to divide and replicate themselves. The replication process is very complicated and occurs with high fidelity. However, many things can go wrong to disrupt replication and therefore multiple controls are in place to prevent replication errors in cells. Most of these controls are associated with growth suppressor signals which are regulated by tumor suppressor genes, such as the p53 protein or retinoblastoma protein (pRB). In normal cells, these signals function to negatively regulate proliferation even when the growth factor signals were triggered. Cancer cells promote their own growth by developing an ability to deregulate these signals through disrupting the function of tumor suppressor genes and modifying extracellular growth signals by signaling to surrounding normal cells to supply cancer cells with excessive growth factors. In addition, cancer cells produce growth factors themselves to which they are reactive to. They promote the level of cell surface receptors that transduce these growth signals, and alter the intracellular signaling networks that translate growth signals into action. Thus, cancer cells can take control of their own destinies.

Avoiding Death

Apoptosis is a biological mechanism that arises when normal cells age or their DNA is too damaged to repair and naturally limits the tumorigenic process. It proceeds via a mechanism that involves permeabilization of the outer mitochondrial membrane with the subsequent release of multiple pro-apoptotic factors into the cytoplasm. Many signals produced by cancer cells, including those indicating DNA damage and elevated levels of proliferative signaling stimulate apoptosis. Cancer cells have shown to develop different strategies to escape apoptosis. The most common is a loss of *p53*, which normally recruits apoptosis. Furthermore, elevated levels of anti-apoptotic Bcl-2 family members, the insulin-like growth factor receptor IGFR, and reduced levels of the FAS receptor are also acquired by the cancer cells to avoid apoptosis.

Limitless Division

Normal cells have a limited potential to divide before they undergo senescence and subsequent cell death. One of the main blocks to sustained replication in cells are specific structures called telomeres, which is a repetitive nucleotide sequence that protect and hold DNA together at the end of the cell's chromosomes. In order to grow unrestricted, cancer cells have

evolved the ability to proliferate without limit, essentially becoming immortal by increasing the expression of the enzyme telomerase, which helps to maintain the telomeres and prevent their shortening.

Stimulating Angiogenesis

Normal cells need oxygen and nutrients brought to the cells by blood vessels, in order to survive and grow. The process of making new blood vessels is called angiogenesis. It is a complex, multistep process involving extracellular matrix remodeling, endothelial cell migration and proliferation, loop formation, capillary differentiation, anastomosis, and finally lumen development [6]. The vasculature is usually quiescent in adult tissue and tightly regulated by the balance of pro- and anti-angiogenic signals in normal tissues. Angiogenesis is only turned on during processes such as wound healing and turned off when the necessity for new blood vessels is met. This process is often deregulated in cancer cells. Cancer cells acquire the ability to recruit blood vessels, in order to provide themselves with nutrients, oxygen, metabolic waste evacuation, sustaining tumor growth and enabling metastatic spreading [7, 8]

Avoiding Immune Destruction

Significant evidence from clinical epidemiology and mouse models has shown that the immune system identifies and eradicates abnormal cells by NK cells or T-cells**.** Conversely, cancer cells have acquired the ability to either escape recognition by the immune system, or develop defensive responses to it.

Reprogramming of energy

Normal cells rely mainly on mitochondrial oxidative phosphorylation to generate the energy needed for their cellular processes. In contrast, in order to fuel their extreme rates of growth and replication, most cancer cells seem to favor glycolysis even in the presence of oxygen as a metabolic program over mitochondrial oxidative phosphorylation. One possible reason for this adjustment is to allow diversion of glycolytic intermediates needed to fuel different biosynthetic pathways that are necessary for proliferation.

Invasion and Metastasis

Normal cells maintain their location in the body, and do not metastasize. Cancer cells acquire mutations that turn on genes which allow them to break free from the primary tumor to penetrate blood vessels and the lymphatic system and then metastasize to other parts of the body. In order to

metastasize successfully, cancer cells reduce cell to cell adhesion and increase cell motility. The most known alteration in cancer cells resulting in invasion and metastasis is in the protein E-cadherin.

Furthermore, enabling characteristics including genomic instability and inflammation have been suggested to be essential for cancer cells to support tumorgenesis.

Genomic mutation in normal cell can either be repaired or the cells undergo apoptosis. However, cancer cells accumulate genetic mutations that are advantageous for tumor growth.

The second proposed enabling characteristic is **tumor-promoting inflammation**. Cancer cells are not only able to evade detection by the immune system and develop defensive response to it but it has been also shown that cancer cells manipulate the inflammatory response such as growth factors for its own purposes to provide the tumor with a source of growth and survival factors. Additionally, inflammatory cells can release ROS, that up-regulate the mutation rates in tumor cells and speed up their progress to unrestricted growth.
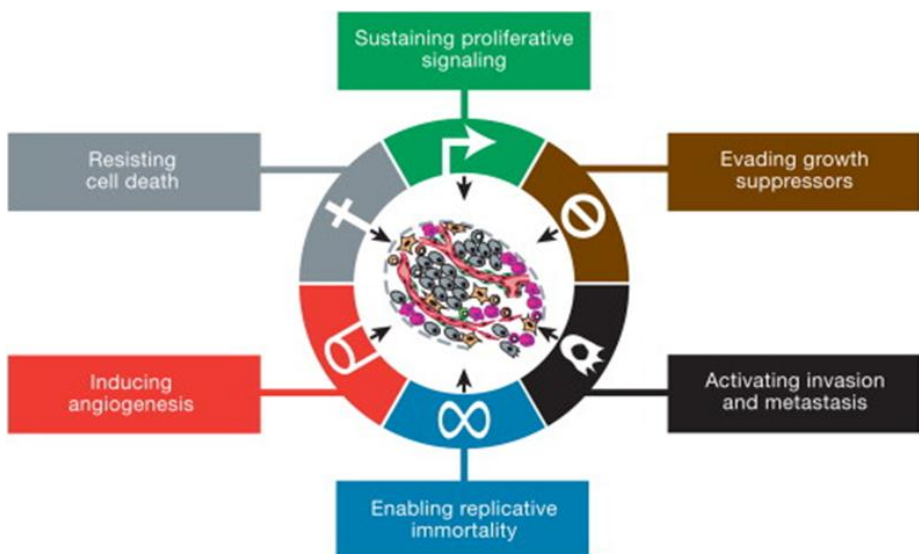
Figure 1. *Hallmarks of Cancer [5]*

1.2   Histopathology of breast cancer

The transition from a normal epithelial cell into a cancer cell is assumed to proceed in a stepwise fashion in the multi-step phenomenon of breast carcinogenesis. Many breast cancers arise from a sequence that begins with an excessive proliferation (hyperplasia), followed by the appearance of breast cells with abnormal characteristics (atypical hyperplasia, AH) which are suggested to increase a women's risk of breast cancer 4-5 fold higher than normal [9]. Subsequent molecular alterations occur in AH, resulting in carcinoma in situ (CIS, noninvasive cancer) [10]. The carcinoma in situ cells acquire a full malignant phenotype, except the ability to invade the surrounding tissues. These cells remain confined within the basement membrane at their site of origin within the terminal duct-lobular unit. There are two types of *in situ* carcinoma including ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS). DCIS is the most common type of noninvasive breast cancer in women. It is usually classified according to the architectural pattern of the lesion (solid, cribriform, papillary, micropapillary and comedo), tumor grade (high, intermediate, and low), and the presence or absence of comedo necrosis [11]. In the high grade DCIS (comedo necrosis), cells tend to grow more quickly and are associated with high grade nuclear and clinically more aggressive behavior [12-14]

In the final stage, the breast cells break through the basal membrane and become an invasive carcinoma. However, not all breast cells certainly follow this progressive pattern and it appears that some cancers may never progress beyond *in situ* disease. It is estimated that 14% to 50% of DCIS cases may progress to invasive cancer if remain untreated [15]. Although it is difficult to predict the percentage of DCIS cases that may progress to invasive cancer and the progression is not fully understood. However, current research suggests that breast carcinogenesis is a series of diverse genetic events that lead to distinct and different pathways headed for invasive carcinoma [16, 17].

1.2.1   Invasive carcinoma

One of the most lethal aspects of breast tumors is their ability to invade the surrounding normal mammary tissue. The cells may then metastasize to other parts of the body through the bloodstream or lymphatic system. Invasive breast tumors are heterogeneous that differ with regard to their histological patterns, biological features and clinical behaviors. Most of invasive breast cancer tumors are adenocarcinomas and are classified according to their appearance under the microscope as ductal or lobular. The difference between invasive lobular (5-15%) and ductal carcinoma (75%) is based on the histological appearance rather than on the site of origin. It is

suggested that both types arise exclusively from the inner, luminal epithelial cell compartment of the terminal-duct lobular unit of the breast. However, most of the breast carcinomas cannot be classified in line with pathological subtypes and are characterized as invasive ductal carcinoma, not otherwise specified (NST). The specified pathological types of breast carcinoma include lobular carcinoma, tubular carcinoma, medullary carcinoma and mucinous carcinoma [18]. Other types of invasive breast carcinoma are inflammatory carcinoma, Paget's disease of the nipple, papillary and invasive cribriform [18].
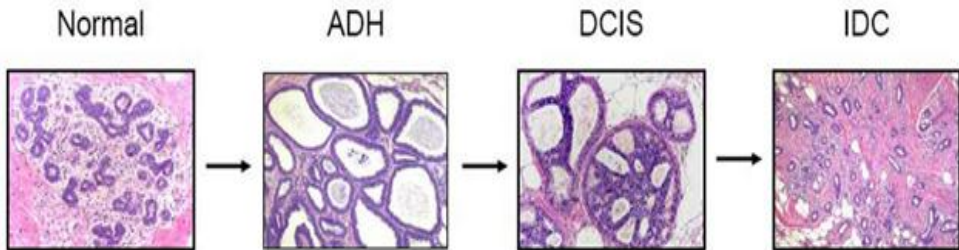

Figure 2. *Classic linear multi-step model of human breast carcinogenesis [19].*

1.3 Prognostic and predictive factors in breast cancer

Traditionally clinical characteristics used to predict prognosis and inform treatment decisions have included age, size, axillary lymph node status, histological grade, hormone receptor status and HER2/*neu* status and these are discussed below:

Tumor grade

The correlation between the morphology, degree of differentiation of breast cancer tumors and clinical outcome was first suggested by Greenhough in 1925 [20]. Owing to the histological complexity of breast cancer, histological grading is considered as a vital component of the pathological assessment of breast cancer. In the early 1990's Elston and Ellis introduced the Nottingham Grading System which is a modification of the Bloom and Richardson grading [21-23]. It is derived from three morphological features including: the tumor mitotic index (rate of cell division), tubule formation (percentage of cancer composed of tubular structures), and nuclear pleomorphism of the tumor cells (changes in nuclear size and uniformity) observed within the tumor. Each of these features is scored from 1 to 3 and

the total of these scores defines the grade; Grade I tumors (score 3 to 5), are well differentiated and associated with a good prognosis, Grade II tumors (score 6 to 7) are moderately differentiated and Grade III tumors (score 8 to 9) are poorly-differentiated and associate with poor prognosis. Tumor grade has been shown to have independent prognostic significance [24]. Patients with grade III tumors have poor prognosis than those with grade I tumors [25].

Table 1. Elston and Ellis modification of Bloom and Richardson grading system of invasive breast cancer

Grading	Score
Tubules	
> 75% of tumor consist of tubules	1
10-75% of tumor consist of tubules	2
< 10% of tumor consist of tubules	3
Nuclear pleomorphism	
Nuclei are small and uniform	1
Moderate variation in nuclear	2
Noticeable variation in nuclear size and shape	3
Mitotic index	
Dependent on defined microscopic field area	1-3
Combined Histological Grade	**Total scores**
Well differentiated, low grade (I)	3-5
Moderately differentiated, intermediate grade (II)	6-7
Poorly differentiated, high grade (III)	8-9

Axillary lymph node status

Approximately 75% of the lymph from the breast travels to the axillary lymph nodes. The axillary lymph node metastasis is considered an important prognostic parameter in treating breast cancer patients. Involvement of an axillary lymph node in breast cancer significantly correlates with unfavorable prognosis compared with axillary lymph node negative tumors [26] and should therefore be treated more aggressively. Various studies have presented a direct relationship between the higher number of axillary nodes involved and clinical outcome [27]. The 5-year survival is reduced from

83% for patients with axillary node negative disease to 73% for 1-3 positive nodes, 45.7% for 4-12 positive nodes, and 28.4% for ≥13 positive nodes [28, 29]. The status of axillary lymph node has been assessed by the surgical sentinel node biopsy procedure to determine if cancer has spread beyond a primary tumor into the lymphatic system [30], followed by a standard axillary node dissection when metastases are present in sentinel nodes [31].

Tumor size

Tumor size is an independent prognostic factor that directly correlates with clinical outcomes [24]. The survival rate was reported to decline with growing tumor size [32]. The 20-year recurrence-free survival rate is 88% in patients with tumors ≤ 10 mm in size, 72% when the tumor is between 21 and 30 mm and 61% in patients with tumors between 31 and 50 mm [33]. Furthermore, an increased tumor size has been correlated with significant axillary lymph node metastasis [34]. The 5-year survival rates for patients with tumors ≤ 20 mm in size with positive axillary nodes have been reported to be around 96%. These rates fall to 45.5% for tumors measuring >50 mm in size [32]. For node-negative patients, tumor size is the most powerful prognostic factor and is routinely used to make adjuvant treatment decisions [35].

Age

The risk of breast cancer is higher in middle-aged and elderly women than in young women [36, 37]. However, patients younger than 40 years old tend to have a more aggressive disease in comparison to older patients [38]. Different studies have suggested that breast cancer in younger women is an indicator of poor prognosis in breast cancer and it is associated with high-grade tumor, axillary lymph node metastasis, and vascular invasion followed by poor clinical outcome. Two large studies reported that breast cancer below the age of 35 years is associated with unfavorable outcome compared to older patients [36, 39]. This may be due to genetic and epigenetic changes [40, 41].

Estrogen receptor (ER)/ Progesterone receptor (PR)

The cDNA encoding an estrogen protein was first cloned and described in 1973 [42]. The name was changed to ER-α when a second form of the receptor, ER-β, was discovered in 1996 [43]. Estrogens are synthesized in the ovary and testis, but also in peripheral tissues via the aromatization of androgens [44]. ER-α is mainly expressed in ovarian stromal cells, hypothalamus and breast cancer cells, whereas ER-β is expressed in endothelial cells, kidney, brain, intestinal mucosa, heart and lungs [45]. PR

is synthesized in the ovary, adrenal gland and during pregnancy by the placenta. PR is an ER-regulated protein and the presence of PR indicates a functional ER pathway. Estrogen and progesterone act through their nuclear receptors ER and PR to regulate transcription of growth factor receptor pathways, which stimulate cell proliferation [46]. Normal breast epithelial cells express very low levels of ER-α and PR, while approximately 70% of all of breast cancer cells express ER and 50% overexpress PR [47, 48]. The expression of ER and PR has been well-known to be one of the most important prognostic factors in breast cancer [49]. Earlier studies reported that ER/PR-positivity correlated with better prognosis including low histological grade, older age of patients, favorable nuclear grade and normal content of DNA [49]. The essential value of ER and PR are their predictive capabilities for response to endocrine therapy with tamoxifen, aromatase inhibitors or ovarian suppression. Tamoxifen acts by binding to the ER and thus hindering the receptor from being activated by estrogens [50]. The aromatase inhibitors disturb the formation of estrogen, in that way improving the inhibition of the ER's pathway, and the ovarian suppression prevent the production of estrogen [51]. Five years of adjuvant tamoxifen declines the risk of recurrence and mortality to 47% and 26%, respectively, of patients with ER-positive tumors [52]. The 5-year survival rates for patients with ER positive tumors have been reported to be around 83%. The rates fall to 62% for patients with ER negative tumors [53].

Human epidermal growth factor receptor 2 (HER2/*neu*)

The HER2/*neu*, is an oncogene that codes for a tyrosine kinase glycoprotein belonging to the family of epidermal growth factor receptor tyrosine kinases (EGFR). This family has four members: HER1 (EGFR), HER2/*neu*, HER3, and HER4. HER2/*neu* stimulates cell growth, cellular differentiation, adhesion and motility and has been shown to be expressed in 15-20% of breast cancer tumors, primarily due to gene amplification [54]. HER2/*neu* is activated by ligand-induced dimerization or receptor pairing [55]. The formation of dimers results in the phosphorylation of specific tyrosine sites, which in turn lead to the stimulation of multiple intracellular molecules. Recruitment of these molecules leads to the activation of diverse downstream signaling systems, such as RAS/MAPK proliferation pathway and/or the PI3K/Akt pro-survival pathway [56]. Amplification and/or overexpression of the HER2/*neu* was reported to be associated with increased tumor aggressiveness, rate of recurrence, mortality, and poor prognosis [57]. A previous study demonstrates that both lymph node metastases and distant metastases, in general overexpress HER2/*neu* protein to the same level as the primary tumor [58]. This constancy of HER2/*neu* expression is of importance when treating breast cancer patients with metastatic disease [58]. HER2/*neu* overexpression has also been associated

with poor clinical outcome when patients were treated with tamoxifen [59]. The HER2/*neu*-targeted therapies are trastuzumab, pertuzumab, and lapatinib. The humanized monoclonal antibody, trastuzumab (Herceptin®, Genentech, CA) which binds to the extracellular domain of the HER2/*neu* and inhibits normal downstream signaling such as proliferation as well as triggering of immune response, is also approved for treating patients with metastatic breast cancer [60]. Comparable to ER/PR status, the value of HER2/*neu* therefore lies in its prediction of targeted therapy response.

1.4 Staging of breast cancer

The prognosis for breast cancer generally depends on its stage, typically graded as I to IV with sub-stages. In the 1940-50's, the first clinical staging system, the Columbia Clinical Classification was developed. In 2002, the International Union against Cancer and the American Joint Committee on Cancer designated staging by a revised TNM to define breast cancer [61]. The TNM system comprises tumor size of the primary tumor (T), lymph node status (N), and the presence or absence of distant metastasis (M). Tumor size is an essential prognostic factor for breast cancer mortality irrespective of other tumor features. The T stages are numbered 1-4, which describe the size of the tumor. The lymph node involvement and the number of affected nodes is the second significant independent prognostic factor that is associated unfavorable prognosis [62]. The N stages are numbered from 0-3, describes the degree of lymph node involvement. Finally, Metastasis is the third factor of clinical importance. Breast cancer patients with distant metastasis have an overall survival of 2 years [63]. The M stages are M0 that describes no sign of cancer spread whereas M1 describes that tumor cells have spread to another part of the body. The TNM staging is related to the clinical prognosis; patients with stage I tumors have a better prognosis compared to patients with stage IV tumors (22, 30, 33). Nearly 90% of cancer patients with grade I survive at least 5 years after diagnosis. Five-year survival rates for grade II and III cancers are 60-80% and 40-50%, respectively, whereas patients with a stage IV cancer have a very poor 10-year survival of 6% [64, 65].

Table 2. **Breast cancer staging system**

Stage	Tumor size (T)	Axillary lymph node (N)	Metastasis (M)	TNM classification
I	<2cm	No	No	T1, N0, M0

II	2-≥5cm	0-3axillary lymph nodes involved	No	T1, N1, M0 T2, (N0 or N1), M0 T3, N0, M0
III	>5cm	≥4 axillary lymph nodes involved	No	T3, N0, M0 T3, N1, M0 T4, Any N, M0
IV	<2cm->5cm	0-≥4	Yes	T (T1, T2, T3), N (N0,N1), M1

Pathologic tumor size was coded as T1 = 0-2 cm, T2 = 2-5cm, T3 = > 5cm, and T4 = ulcerated or attached to skin or muscle; Axillary lymph node status was coded as N0= negative and N1= positive; Metastasis was coded as M0 = no metastasis of tumors and M1 = tumor has metastasized.

1.5 Molecular subtypes of breast cancer

In the early 2000's, five intrinsic molecular subtypes of breast tumors with distinctive gene signatures have been identified using gene expression microarray data [66]. These intrinsic subtypes include basal-like, HER2/*neu* -enriched, luminal A, luminal B, and normal-like tumors [67]. Each of these subtypes has diverse risk factors for incidence, risk of progression, and organ sites of metastases [68]. These molecular subtypes show variable prognosis and response to therapy, therefore they have been suggested to originate from different cell types and follow different progression pathways. Luminal A tumors are frequently of low histological grade and have good clinical outcomes, show high levels of expression of ER-related genes and low levels of proliferative genes [69]. Luminal B tumors have been shown to have expression of ER-related genes, to be often of higher grade with higher expression of proliferative genes, exhibit *P53* mutation, and poor survival outcome compared to patients with luminal A [69]. Furthermore, *BRCA2-*mutated tumors are frequently classified as luminal B [70]. The HER2/*neu* enriched subtype of breast cancer is defined by amplification/ overexpression of HER2/*neu* related genes and lack of expression of ER-related genes [69]. Similar to luminal B tumors, HER2/*neu*-enriched tumors are often of higher grade and 50% of them show *P53* mutation [71]. However, breast tumors with amplified/overexpressed HER2/*neu* are regarded as luminal B subtype, if they express estrogen-related genes [72]. Basal-like tumors, defined by the expression of genes usually found in normal basal cells located in the epithelial layer of the mammary gland, such as cytokeratin 5, cytokeratin 17 and EGFR [73-75]. The majority of basal-like tumors are triple negative that lack the expression of ER, PR and HER2/*neu* [74]. In addition, 80% of *BRCA1*-mutated tumors belong to the basal-like subtype [76]. Basal-like tumors are usually of high histological

grade, highly proliferative, and generally show mutations in both p53 and pRB protein function [77]. Furthermore, basal-like tumors have been associated with poor clinical outcome due to improved invasiveness and formation of distant metastasis [67]. At present, there is no molecular-based targeted therapy for ER-, PR- and HER2/*neu*-negative tumors, and only approximately 20% of these tumors respond well to standard chemotherapy.[68]. In addition, as gene expression studies progress, additional sub-classifications of breast tumors are likely to take place. Indeed, two additional molecular subtypes, referred to as claudin-low and molecular apocrine were identified [78, 79]. The majority of claudin-low tumors are characterized by lack of ER, PR, HER2/*neu* expression, and loss of genes involved in cell-cell adhesion. They are of higher-grade and are enriched for mesenchymal and stem cell-like biological processes [80].The molecular apocrine tumors defined by lack of expression of ER-related genes, with increased androgen signaling and share some characteristics with HER2/ne*u* enriched tumors.

2 AIM

Efforts continue to identify and validate potential biomarkers for breast cancer and to improve breast cancer risk prediction models. The overall purpose of this thesis was to add to this effort.

In **Paper I** the aim was to characterize the essential regulatory pathways for psoriasin expression.

In **Paper II** the aim was to investigate the effect of psoriasin expression on endothelial cells.

In **Paper III** the aim was to identify novel prognostic biomarkers for breast cancer by investigating the prognostic value of the candidate biomarkers CCNB2, ASPM, CDCA7, KIAA0101, and SLC27A2 in breast cancer.

The aims of **Paper IV** were to determine the expression pattern and correlation of GGH, FAAH, PIR and TAF5L with clinical outcomes as well as classical clinicopathological characteristics in invasive breast cancer patients.

3 MATERIALS AND METHODS

Materials are described in the respective papers. The methods used in this thesis are well-known and what follows is a short summary of the materials and methods:

Cell lines

Normal breast epithelium (MCF10A; Paper I and II)
Mammary breast carcinoma (MDA-MB-468; Paper I and II)
Human umbilical vein endothelial cells (HUVEC; Paper II)
Neonatal human dermal microvascular endothelial cells (HMVEC-d; Paper II)

Tumor material

Eleven formalin-fixed, paraffin-embedded (FFPE) DCIS tissues were used for immunostaining (Paper I). Eighty FFPE and fresh-frozen primary invasive breast tissues were used for immunostaining and quantitative PCR (RT-PCR) analysis (Paper III and IV). The DCIS and primary invasive breast tissues were obtained from the Departments of Pathology and Oncology at Sahlgrenska University Hospital in accordance with the Declaration of Helsinki and approved by the Medical Faculty Research Ethics Committee (Gothenburg, Sweden). The clinicopathological characteristics of the tumors are given in Paper I, III and IV. The selected serial analysis of gene expression (SAGE) libraries in Paper I were essentially as described elsewhere as part of the National Cancer Institute Cancer Gene Anatomy Project (CGAP) [81]. The SAGE Genie website (http://cgap.nci.nih.gov/SAGE) provides a quantitative view of the gene expression of selected genes in many different human tissues. In brief, The SAGE libraries included freshly-frozen 6 normal breast tissues, 8 DCIS tissues of which 5 were high-grade, comedo DCIS and 3 were intermediate-grade with no necrosis, 9 invasive breast tissues and 3 metastatic breast tissues were obtained from the Brigham and Women's Hospital, Massachusetts General Hospital, and Faulkner Hospital (all Boston, MA), Duke University (Durham, NC), University Hospital Zagreb (Zagreb, Croatia), and the National Disease Research Interchange. Sex of the DCIS tissues were derived from patients with concurrent invasive breast carcinomas whereas the others were pure DCIS.

Transfection methods:

Short hairpin RNA (Paper I).

The Short hairpin RNA (shRNA) is a RNA molecule that contains a complementary sense and antisense fragments corresponding to the target gene and between them there is a short loop. The shRNA was cloned into a plasmid, which then are cleaved by the RNase III family member, Dicer, into smaller pieces of 21-23 nucleotides known as siRNA corresponding to both sense and antisense strands of the target gene. Dicer offers the siRNAs to a group of proteins called the RNA- Interference Silencing Complex (RISC). The RISC uses the antisense strand of the siRNA to bind to and degrade the corresponding mRNA, resulting in gene silencing. Stable MCF10A clones expressing shRNA for human ICAM-1 and psoriasin respectively were produced using Lipofectamine.

Infection with recombinant retrovirus (Paper I and II)

The retrovirus is a RNA virus. It contains a RNA-dependent DNA polymerase (a reverse transcriptase) that directs the synthesis of a DNA form of the viral genome after infection of the host's genome. The integrated DNA can then be inherited across generations. The MCF10A cell line was infected with a recombinant retrovirus overexpressing psoriasin. The retroviral protein expression was confirmed with western blotting.

Expression methods

Quantitative Real-Time PCR (qRT-PCR) (Paper I, II, III and IV)

QRT-PCR was used for cDNA quantitation analysis in order to monitor the mRNA expression patterns of the genes of interest. In order to detect the amplified PCR products in real time, the reaction included a fluorescent molecule that reports an increase in the amount of the amplified product with a proportional increase in fluorescent emission. The fluorescent molecules commonly used are non-specific DNA-binding dyes and fluorescently labeled sequence specific primers or probes. Subsequently, the exponential accumulation of PCR products in each cycle is detected directly by monitoring the increase in fluorescence of the dye.

Western blotting (or immunoblotting) (Paper I, II, and IV)

Western blotting is a qualitative and a semiquantitative method used to detect target proteins. The sample proteins are separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE) that provides information about molecular weight and the potential existence of different isoforms of the target proteins. Followed by, immobilization of sample proteins on

synthetic membranes. The detection is then performed by enzyme-labeled antibodies correspond to the genes of interest.

Immunohistochemistry (IHC) (Paper I, III and IV).

IHC is a commonly used method for examination of protein levels in tissues. In brief, an antibody corresponding to an epitope on a protein of interest is immunized with the FFPE tissues that will be investigated. A secondary antibody conjugated with horseradish peroxidase is then add followed by diaminobenzidine (DAB), which produces a crisp brown color when oxidized by peroxidase. The nuclei are then counterstained with haematoxylin, which provide a clear blue color.

Fluorescence in situ Hybridization (FISH) (Paper III and IV).

In brief, fluorescently labeled BAC clones were hybridized to known DNA sequences in either interphase or metaphase cells. Using an epifluorescence microscope, the fluorescence from each BAC clone can then be registered.

4 RESULTS AND DISCUSSIONS

4.1 Paper I

Previous studies have shown that the overexpression of psoriasin in mammary epithelial cells is induced by anoikis (suspension culture), prolonged cell confluency [82] and ROS [83]. All of these conditions may mimic high-grade DCIS *in vivo*, when the epithelium hyper-proliferate and lose contact to their basement membranes. Interestingly, cytokine interferon-gamma IFN-γ was found to down-regulate the expression of psoriasin in suspension culture while it had no effect on psoriasin expression in confluent cells or ROS treated cells [84]. These observations suggest that the overexpression of psoriasin in suspension cultures may due to loss of adhesion signaling, and IFNγ potentially interact with psoriasin regulating adhesion signaling that led to psoriasin suppression. Normal epithelial cells require adhesion to the extracellular matrix (ECM) for survival while tumor cells frequently demonstrate a decrease in cell to matrix adhesion. Many studies have shown that alterations in the cellular microenvironment caused by deregulation of several classes of proteins including extracellular proteases, cadherins, cell-cell adhesion molecules (CAMs) and integrins associate with tumor invasion and metastasis [85]. In Paper I, we revealed that the expression of psoriasin protein was not induced when the binding of some integrin receptors which recognize their ligands by RGD sequence (Arg-Gly-Asp) was blocked by the RGD-competitive ligand inhibitor. This finding suggests that integrins recognizing this sequence do not regulate the expression of psoriasin expression by ECM contact. The IFNγ is a well-known inducer of adhesion molecules. We investigated the role of 13 IFNγ-stimulated adhesion molecules that may play a role in the regulation of psoriasin including Activated leukocyte cell adhesion molecule (ALCAM), Ras homolog gene family member C (ARHC), Cadherin 5 (CDH5), CD47, Claudin 5 (CLDN5), Desmoglein 1 (DSG1), Intercellular adhesion molecule 1 (ICAM-1), Interferon induced transmembrane protein (IFITM1), Integrin alpha 2 (ITGA2), Kallmann syndrome 1 sequence (KAL1), Selectin L (SELL), Thrombomodulin (THBD) and Thrombospondin 1 (THBS1) [83]. The expression of psoriasin and the 13 IFNγ-stimulated adhesion molecules were analyzed in normal breast tissue and DCIS tumors using the SAGE database available from the CGAP website. We found that IFNγ-stimulated adhesion molecules ICAM-1 and THBS1 are negatively associated to psoriasin expression in normal and DCIS specimens. In DCIS tissues, the expression of psoriasin was elevated, while the levels of ICAM-1 and THBS1 were down-regulated compared with normal breast tissues which suggest that ICAM-1 and THBS1 are possibly involved in the regulation of psoriasin expression. Previously, we reported that the expression of psoriasin, calgranulin-A (S100A8) and

calgranulin-B (S100A9) share the same signaling pathways [82]. Therefore, we further analyzed the expression of 34 well-known adhesion molecules, in addition to psoriasin, calgranulin-A and calgranulin-B in normal and DCIS SAGE libraries in order to investigate the effect of the selected adhesion molecules on the expression of the three S100 proteins. The results indicated that the expression of one of the selected adhesion molecules, the tumor-associated mucin1 (MUC1), which is a ligand for ICAM-1 was positively correlated to the expression of psoriasin, calgranulin-A and calgranulin-B proteins. Based on these findings, we focused our interest on ICAM-1 and MUC1 as potential regulators of psoriasin expression. Interestingly, the expression of MUC1 was up-regulated in DCIS specimen in comparison to normal tissue. Previous studies have reported that the expression of MUC1, which is a transmembrane glycoprotein, was associated with poor prognosis and unfavorable clinical outcome in breast cancer [86, 87]. In addition, both MUC1 and psoriasin were found to associate with increased survival in response to oxidative stress and to be regulated by the NF-κB pathway [83, 88]. Notably, we found that similar to psoriasin, calgranulin-A and calgranulin-B, the levels of MUC1 protein were also up-regulated in suspension cultures of MCF10A cells, whereas the expression of ICAM-1 protein was downregulated. These findings suggest that ICAM-1 may regulate the expression of psoriasin. We showed that the down-regulation of ICAM-1 expression by short hairpin RNAs (shRNA) in the epithelial MCF10A cells led to the elevated levels of psoriasin, calgranulin-A, calgranulin-B and MUC1 protein. Furthermore, we investigated the mechanism for the up-regulation of psoriasin in MCF10A cells with decreased protein level of ICAM-1 by shRNA. We have previously demonstrated that psoriasin is induced by ROS and down-regulated by the antioxidant NAC [83]. We therefore treated MCF10A with down-regulated ICAM-1 by shRNA cells with NAC and we also measured their ROS production compared to MCF10A control cells. We found that the antioxidant NAC abolished the level of psoriasin in MCF10A control cells. However, the levels of psoriasin were still detected when MCF10A cells with reduced ICAM-1 by shRNA were treated with NAC. Moreover, we showed that the intracellular levels of ROS generation were not elevated in MCF10A cells with reduced ICAM-1 by shRNA compared with MCF10A cells. These findings suggest that signals other than ROS may be involved in the up-regulation of psoriasin in this condition. The binding of MUC1 to ICAM-1 has been reported to induce intracellular calcium signaling, mediated by the phospholipase C (PLC)-IP3 pathway [89]. Interestingly, we found that PLC-IP3 inhibitors, U73122 or 2-APB, abolished the expression of psoriasin in MCF10A cells with reduced ICAM-1 by shRNA. We also demonstrated that the expression of psoriasin was elevated in MCF10A cells when the cells were treated with PLC-activator m-3M3FBS. Functionality of m-3M3FBS, and U73122 was demonstrated by phosphorylation of PLCγ1, confirming an active signaling pathway.

4.2 Paper II

Growth of tumors and metastasis are processes known to require neovascularization. The DCIS tumors are avascular but like normal tissue, require oxygen and metabolites and gaining access to the host vascular system is essential for tumor progression and metastasis. Therefore, angiogenesis has been intensively studied in the context of cancer growth. It is a complex multistep process involving extracellular matrix remodeling, endothelial cell migration and proliferation, loop formation, capillary differentiation, anastomosis and finally lumen development. The vascular endothelial growth factor (VEGF) is known as a multifunctional cytokine that play a critical role in blood vessel formation including both vasculogenesis and angiogenesis [90]. Overexpression of VEGF has been considered as the major factor underlying pathological angiogenesis *in vivo* in conditions such as psoriasis, macular degeneration, and tumor proliferation [91]. The group has previously shown that the down-regulation of endogenous psoriasin expression by shRNA in the MDA-MB-468, a metastatic breast carcinoma cell line, inhibited tumor growth and down-regulated the expression of VEGF *in vivo* [92]. This finding suggests that psoriasin may increase tumor growth in *vivo* by promoting angiogenesis. In accordance with this, high-grade DCIS, which commonly over-express psoriasin, was found to be correlated with increased VEGF levels and angiogenesis [93]. In Paper II, the retroviral mediated stable overexpression of psoriasin, adenoviral mediated transient overexpression of psoriasin and anoikis, were studied using the epithelial immortalized non-tumor-derived cell line MCF10A, in order to investigate the effect of the up-regulation of psoriasin on VEGF expression. We demonstrated that elevated mRNA expression of psoriasin led to the significant up-regulation of VEGF mRNA level. This finding suggests that both exogenous and endogenous psoriasin overexpression is associated with increased VEGF expression in mammary epithelial cells. Next, we suppressed the low endogenous level of psoriasin in MCF10A cells with shRNA targeting psoriasin mRNA. We showed that the down-regulation of psoriasin by shRNA led to the reduction of the expression of VEGF mRNA in MCF10A cells treated with H_2O_2, a stimulus known to induce high endogenous level of psoriasin. Psoriasin is secreted but also located in the cytoplasm and the nucleus of the cells expressing it [82, 94]. We demonstrated that the extracellular recombinant psoriasin protein significantly prompted endothelial cells proliferation compared to the control untreated cells, and was comparable to that seen for VEGF-stimulated cells. No significant change in proliferation was seen when endothelial cells were infected with psoriasin-expressing adenoviruses. Furthermore, we demonstrated that, opposite to epithelial cells, psoriasin was neither expressed nor inducible in endothelial cells. These findings led to the hypothesis that psoriasin secreted from epithelial cells may interact

with a specific receptor on the surface of endothelial cells, which may in turn induce endothelial cell proliferation and angiogenesis. Previous studies have shown that the receptor for advanced glycation end products (RAGE) has been demonstrated to be expressed in endothelial cells [95] and to be the putative receptor for several S100 proteins [96, 97]. We therefore predicted that psoriasin may be a putative ligand to RAGE in endothelial cells. To examine this, we used sRAGE, a truncated form of the receptor spanning the extracellular domain of human RAGE, to prevent the putative interaction between psoriasin and the cell surface receptor RAGE. By blocking RAGE-psoriasin interactions, we indicate a significant suppression in endothelial cell proliferation and tube formation. In addition, we showed that the mRNA as well as the protein levels of RAGE was significantly up-regulated in endothelial cells treated with recombinant psoriasin protein. These findings suggest that psoriasin stimulates endothelial cell proliferation through the receptor RAGE. Several studies have reported that both psoriasin and VEGF are induced by ROS [83, 98] and low levels of ROS may induce proliferation of different cell types and specifically endothelial cells [99]. Furthermore, the expression of S100A8 and S100A9 was previously reported to elevate the intracellular levels of ROS generation [100]. Interestingly, we showed that psoriasin induce ROS in endothelial cells in the same range as that previously demonstrated for S100B [101]. The ROS generation was reduced significantly in MCF10A cells with suppressed psoriasin expression by shRNA. These findings suggest that psoriasin may induce low levels of ROS by itself, leading to a further increase in ROS levels, VEGF expression and endothelial cell growth. Previous studies reports that RAGE transduces inflammatory responses and plays a role in the pathogenesis of several diseases including neurodegeneration, inflammation, and cancer [102, 103]. Interestingly, we found that sRAGE significantly eliminated ROS generation in endothelial cells after treatment with psoriasin, further suggesting that RAGE acts as a receptor for psoriasin. Furthermore, we found that the anti-oxidant Bcl-2 significantly decreased the effect of recombinant psoriasin on endothelial cell growth, suggesting that psoriasin protein induces ROS generation.

4.3 Paper III

Breast cancer is a potentially fatal malignancy in females despite the improvement in therapeutic techniques. The identification of novel molecular signatures is an essential need for earlier detection, predicting prognosis and monitoring effects of treatment. We have previously used microarray analysis and identified molecular gene signatures associated with aggressive breast cancer [104]. In Paper III, we selected CCNB2, ASPM, CDCA7, KIAA0101 and SLC27A2 included in these gene signatures based on their significantly deregulated gene expression according to short-term

disease-specific survival, triple-negative status, and/or or stratified according to histological grade as defined by Bloom, Richardson, Elson/Ellis (BRE) grading system [23]. We investigated the prognostic value of the selected candidate biomarkers using an independent cohort of 80 primary invasive breast tumors. The *CCNB2* gene is located at 15q22.2 and is a member of the B-type cyclin family, including cyclin B1 and B2. It is an essential regulator of the cell cycle and plays an important role in regulation of transcription, DNA repair, differentiation, and apoptosis. *CCNB2* is involved in the G2-M transition in eukaryotes by activating CDC2 kinase and its inhibition induces cell cycle arrest [105-107]. In agreement with a crucial role in cell growth, several studies detected overexpression of CCNB2 in human tumors, including lung, colorectal adenocarcinoma, and pituitary adenomas [108-111]. Serum circulating *CCNB2* mRNA levels were found to be higher in lung and digestive tract cancer patients compared to normal controls and were correlated with cancer stage and metastasis status [112]. Furthermore, the *CCNB2* gene was included in a set of genes detected in node-negative breast tumors associated with poor prognosis [113]. In Paper III, we showed that the expression of CCNB2 protein was significantly up-regulated in 92% of breast tumors from short-term survivors in comparison with 52% of long-term survivors ($P<0.001$). The expression of CCNB2 exhibited a lower disease specific survival (DSS) probability with a 6 fold higher risk of mortality. Our results suggest that CCNB2 has as oncogenic potential and its overexpression may give some proliferative advantage. The expression of the CCNB2 protein was also studied in relation to the traditional clinicopathological parameters HER2/*neu*, ER/PR status, axillary lymph node status, tumor size, and tumor grade. We have shown a significant correlation of CCNB2 protein expression with breast tumor type ($P=0.04$). The multivariate analysis including CCNB2 and several clinicopathological parameters verified that CCNB2 is an independent prognostic indicator for DSS, as presented by the fact that hazard ratio (HR) for CCNB2 adjusted for other clinicopathological features remained unaffected and significant $P<0.001$ for DSS. These data indicate that tumors with histological grade (I, II and III), axillary lymph node status (positive, negative), tumor size (0-2, 2-5, and >5), ER/PR status (positive, negative) and HER2/*neu* status, exhibiting CCNB2 protein expression, have a more unfavorable prognosis, with an increased risk of shorter disease specific survival rates. Moreover, the predictive power of CCNB2 in addition to the clinicopathological parameters model was slightly higher (C-index = 0.795) compared to the lower C-index of 0.698 for the model including all clinicopathological parameters alone. Thus, the accuracy in patient prognosis may be improved by measuring CCNB2 expression in cases of breast cancer. The ASPM was reported to participate in spindle organization, spindle orientation, mitotic progression, and cytokinesis [114-117]. The overexpression of ASPM protein was detected in several cancer forms [116, 118-121] while its knockdown inhibits tumor proliferation [118]. In this study, we observed that 69% of the

analyzed tumors expressed ASPM protein in the nucleus of the cell. A significant correlation between CCNB2 and ASPM (*P*=0.03) was seen. Up-regulation of CCNB2 and ASPM was previously detected in glioblastoma multiforme xenograft tumors and *de novo* glioblastoma multiforme tumors [122]. Activation of *CCNB2* and *ASPM* genes induces tumorigenic phenotypes in a number of cancers, whereas their inhibition abrogates cellular proliferation in mice and induces genomic instability [107, 123]. The *CDCA7* gene has been involved in neoplastic transformation and it is one of the downstream targets of the *Myc* oncogene [124]. We found that the nuclear expression of CDCA7 was expressed in almost all the analyzed primary invasive breast tumors. The deregulation of cell cycle control is a vital feature of cancer pathogenesis, therefore observed overexpression of CDCA7 protein in almost all studied tumors was predictable. KIAA0101 is mainly expressed in mitochondria and partially in nuclei, playing an essential role in the regulation of DNA repair, cell cycle progression, and cell proliferation [125]. Moreover, the *KIAA0101* gene was reported to be over-expressed in tumors of the esophagus [126], colon [127], lungs [128, 129], and breast [130]. The expression of KIAA0101 was observed in 79% of the immunostained tumors. The elevated expression levels of KIAA0101 were confirmed by real time qRT-PCR, in 92% of the studied tumors. Breast tumors are heterogeneous with multiple cell types within the tumor and in the surrounding microenvironment (including cancer-associated fibroblasts, stromal cells, endothelial cells, pericytes, immune cells, etc.) [131]. In Paper III, total RNA was extracted from the bulk tumor, without performing any cell selection prior to qRT-PCR analysis. Therefore, the possibility of tissue heterogeneity, accounting for the discordance between mRNA and protein expression cannot be excluded expression cannot be excluded. In addition, there was no association between elevated ASPM, CDCA7 and KIAA0101 protein levels and DSS or any other clinical parameters. Consequently, ASPM, CDCA7 and KIAA0101 may be involved only in tumor initiation. We observed discordant results between mRNA and protein expression of SLC27A2. High mRNA expression was detected in 83% of the analyzed tumors, but protein expression was only seen in 25%, possibly owing to tissue heterogeneity, posttranscriptional regulation and differences in mRNA and protein turnover rates [132, 133]. These findings suggest that down-regulation of SLC27A2 protein expression in the analyzed tissues may contribute to disease progression. Indeed, this gene was previously reported to control the tumor suppressor gene PARP and reduced SLC27A2 expression levels were found in the metastatic compared to the non-metastatic neuroendocrine tumors [134]. However, no significant difference could be seen on the effect of SLC27A2 protein expression on DSS in breast cancer, nor could any association between the protein expression of SLC27A2 and the conventional clinical characteristics be observed. Furthermore, no correlation between the expression of CCNB2, SLC27A2, KIAA0101, and CDCA7 was seen.

4.4 Paper IV

Previously, we performed gene expression analysis in 43 axillary lymph node negative tumors [135]. Our analysis showed a critical role of 51 genes whose persistently deregulated mRNA levels were significantly associated with unfavorable clinical outcome. In Paper IV, four candidate biomarkers, *GGH, FAAH, PIR* and *TAF5L* were selected among the identified 51-gene signature. Importantly, the expression of the candidate biomarkers *GGH, FAAH, PIR* and *TAF5L* were also reported to be correlated with unfavorable clinical outcome in a data sets of 78 node-negative breast tumors [113]. In addition, several publications reported their involvement in various cancer forms [136-139]. In Paper IV, we demonstrated new information supporting a role of GGH expression in invasive breast cancer. The cytoplasmic expression of GGH protein among the tumor tissues was detected in 75% (54/72) of the cases. Nineteen percent of non-cancerous breast tissues exhibited GGH positive expression, while the remaining tissues had negative staining for GGH (χ^2=17.9, *P*<0.001). A previous study reported that up-regulation of GGH protein was also detected in urothelial carcinoma of the bladder in comparison with non-cancerous cells [136]. In addition, tumoral GGH protein expression was significantly up-regulated in high histological grade tumors in comparison with low histological grade tumors (*P*<0.001). High expression of tumoral GGH was also observed to be significantly associated with ER/PR status (*P*<0.001). Taken together, these finding suggest that the expression of GGH is associated with invasiveness and GGH may increase as the disease progresses. However, detected GGH protein expression in the non-cancerous tissues may represents the normal function of GGH in maintaining tissue homeostasis or may predict progression of premalignant lesions [140]. Eight-year survival of patients with no or lower expression of GGH was significantly better than those with a higher expression (*P*=0.032). Indeed, 8-year DSS rate was 39% among patients with GGH expressing tumors compared to 68% among patients whose tumors were GGH-negative. Furthermore, the univariate Cox proportional hazards regression analysis revealed that GGH expression exhibited a lower DSS probability with a 2.5 fold higher risk of death (95% CI: 1.0-2.5; *P*=0.04). In addition, the multivariate analysis verified that GGH is an independent negative factor in predicting patient DSS as presented by the fact that HR for GGH adjusted for age, histological type, histological grade, ER/PR status, HER2/*neu* status, pathologic tumor size, and axillary lymph node status remained significant (HR=3.6, *P*= 0.01, 95 % CI: 1.3–10.3). These findings suggest that GGH may be involved in promoting carcinogenesis. Furthermore, the elevated levels of GGH were found to be correlated with shorter recurrence free survival (RFS) with more than 35 fold increased risk (95% CI: 0.43–2932, *P*=0.009). The 8-year RFS rate was 100 % in GGH- negative tumors, while it dramatically decreased to 10% in GGH

expressing tumors suggesting that GGH expression may predict the recurrence behavior of breast cancer. Notably, the association between GGH expression and different cancer forms has been previously reported. The elevated levels of GGH were reported to be correlated with poor clinical outcome in pulmonary neuroendocrine tumors [141]. Elevated plasma level of GGH was observed in patients with metastatic breast cancer in comparison to control subjects and to patients whose cancer was in remission [142]. High GGH expression level was also detected in hepatoma cells compared with rat hepatocytes [143]. Furthermore, GGH expression was found to act as a prognostic biomarker for acute leukemia in response to methotrexate therapy [144]. Consistent with these findings, our data further support that the dysfunction of GGH may play an important role in breast cancer progression and GGH may be an amenable therapeutic target in breast cancer. To further confirm the results of IHC, we assessed GGH mRNA expression levels by qRT-PCR. In tumor tissues, the mRNA expression of GGH was increased specifically in patients with short-term survivor. This increase corresponded to protein accumulation based on IHC data, indicating transcriptional activation (t-test, $P= 0.023$). However, the *GGH* gene may be also regulated in tumors at posttranscriptional levels, since the GGH protein in 5 of 62 invasive breast cases was elevated, whereas no increase in mRNA was observed in these samples by qRT-PCR. The expression of GGH was further confirmed by western blot analysis in 7 representative patients. Interestingly, two closely spaced bands corresponding to GGH protein expression were detected at 33- and 37-kD. Similar observation was previously reported [145], which suggests a post-translational modification of the protein. The cytoplasmic expression of FAAH was significantly up-regulated in invasive breast tumor tissues compared to the non-cancerous tissues. Four percent of the FAAH protein expression was positive in non-cancerous breast tissues whereas 89% of the breast cancer tissues expressed FAAH (χ^2=19.3, $P<0.001$). In addition, the expression levels of FAAH were significantly increased in patients with higher number of axillary lymph node metastases (P=0.023). Up-regulation of FAAH indicates down-regulation of cannabinoids, which play an important role in preventing tumor growth [146]. Taken together, these findings suggest that the elevated level of FAAH may down-regulate the levels of cannabinoids and thus promote breast cancer tumors invasion and metastasis. Interestingly, a significant correlation between GGH and FAAH protein expressions was detected in the tumor ($r = 0.31$, $P= 0.02$). In tumor tissue samples, seventy-one percent of the tumors had positive expression of GGH and FAAH simultaneously. Seven percent of the tumors had negative expression of GGH and FAAH at the same time. No association in the non-cancerous tissues was seen, suggesting that the tumor micro-environmental effects may regulate the expression of GGH and FAAH simultaneously [147]. In addition, GGH accumulation may reflect a functional correlation with FAAH expression, which could play a key role in the progression of

breast carcinoma. The frequency and levels of PIR expression was similar between non-cancerous and invasive breast cancer tissues which might be relatively a consequence of sharing the same microenvironment. Eighty-six percent of non-cancerous breast tissues and 85% of the breast cancer tissues were positive for PIR expression. Previous studies reported that some gene expression patterns in the invasive tissues are comparable to their non-cancerous breast tissues, suggesting that these signatures may predict progression of early premalignant lesions [140, 148, 149]. The PIR functions as a transcriptional regulator whose expression was reported to be deregulated in several cancer types. High expression of PIR was reported to be essential to overcome the senescence barrier [150]. We also examined the clinical significance of PIR protein expression. The higher expression of PIR was significantly associated with presence of lymph node metastasis, suggesting that the expression of PIR is associated with invasiveness and supports the reported association of PIR expression with enhanced malignant potential [150, 151].The TAF5L protein was highly expressed in the cell nucleus of the non-cancerous cells compared to the adjacent cancerous cells in the analyzed specimens (χ^2=28.2, $P<0.001$), which suggests a potential tumor suppressor role in breast cancer. The expression of TAF5L was elevated in patients with low histological grade tumors compared to patients with high histological grade tumors, although the differences were not significant (P=0.06). Furthermore, high mRNA expression levels of TAF5L were detected in 97% of the analyzed tumors, whereas only 56% of the analyzed tumors expressed TAF5L protein. The reduction of TAF5L protein in the analyzed tissues may contribute to disease progression. In this cohort of patients, even though the expression of FAAH, PIR and TAF5L did not predict DSS and RFS, the expression of these candidate biomarkers were significantly associated with other clinicopathological characteristics.

5 CONCLUSIONS

The present thesis described all the important incremental steps made in achieving the aims within the timeframe of the four-year PhD. The conclusions for the different papers are as follow:

Paper I

- Our results suggest that the reduction of ICAM-1 expression in mammary epithelial cells may contribute to the elevated levels of psoriasin expression in high-grade DCIS tumors and to the stimulation of MUC1 expression.
- Our findings suggest that psoriasin is an intracellular calcium-dependent target of the PLC pathway.

Paper II

- Our results suggest that psoriasin contributes to the expression of ROS and VEGF and acts through RAGE to promote endothelial cell proliferation.
- Our data raise the possibility that psoriasin may be evaluated as a novel anti-angiogenic target in breast cancer.

Paper III

- Our results suggest that CCNB2 is a potential independent prognostic factor that may be useful in conjunction with other clinicopathological features in breast cancer.
- We have shown that CCNB2 expression represents a threshold that can stratify breast cancer patients in a high risk group associated with an increased risk of mortality when compared to 8-year survivors.

Paper IV

- Our results suggest that elevated expression of GGH protein is associated with unfavorable prognosis and poor outcome in patients with invasive breast cancer.
- Our data suggests that GGH is a potential independent prognostic factor of DSS when compared to other widely used prognostic factors.
- We have also demonstrated an association between elevated levels of FAAH and PIR and high number of axillary lymph node involvement and lymph node metastasis, respectively.

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Where there is a will there is a way